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The blood level of transforming growth factor-β rises in the early stages of acute protein and energy deficit in the weanling mouse

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Plasma transforming growth factor (TGF)-β levels are high in the advanced stages of acute (wasting) pre-pubescent deficits of protein and energy. Consequently, this potently anti-inflammatory cytokine may help to sustain the depression of inflammatory immune competence in acute malnutrition. Our objective was to determine if plasma TGF-β levels rise during the early stages of acute malnutrition and, secondarily, to confirm the elevation reported previously in advanced weight loss. In two experiments, male and female C57BL/6J mice, initially 19 d old, consumed *ad libitum* a complete purified diet (group C), or in restricted daily quantities (group R) or had free access to an isoenergetic low-protein diet (group LP). TGF-β bioactivity in platelet-poor plasma was determined via inhibition of Mv1Lu mink lung cell proliferation after 3 d (Expt 1, early stage) or 14 d (Expt 2, advanced stage) of dietary intervention. At 3 d, mean plasma TGF-β bioactivities were 802 (C), 2952 (R) and 4678 (LP) pg/ml, and after 14 d mean bioactivities were 1786 (C), 5360 (R) and 5735 (LP) pg/ml. At both time points, the malnourished groups differed from age-matched controls ($P \leq 0{\cdot}05$). Thus, metabolically distinct weanling systems mimicking paediatric marasmus (group R) and kwashiorkor (group LP) exhibit an early rise in blood TGF-β concentration, and this cytokine joins corticosterone and IL-10 as a third anti-inflammatory hormone temporally positioned to contribute to the initiation (and maintenance) of malnutrition-associated immune depression. This investigation contributes new insight into the active anti-inflammatory form of immune competence that appears to prevail in acute pre-pubescent malnutrition.

Cytokines: Mice: Protein–energy malnutrition: Transforming growth factor-β

A defining characteristic of acute (i.e. wasting) pre-pubescent protein and energy deficit is depressed inflammatory immune competence, although the aetiology of this aspect of malnutrition pathology is poorly understood[1]. A recent proposition suggests that this immunological phenomenon represents a regulated pathophysiology, controlled by hormones and cytokines, rather than a biologically trivial disintegrative loss of immunological control[1]. In this connection, murine models that closely mimic acute paediatric malnutrition exhibit elevations in blood levels of three potent anti-inflammatory mediators, namely corticosterone[2], IL-10[3] and transforming growth factor (TGF)-β[3], in the most advanced stages of wasting pathology. Moreover, in the same pre-pubescent experimental systems, blood levels of both corticosterone[2] and IL-10[4] rise in the early stages of weight loss. Importantly, high blood levels of TGF-β are also reported in the advanced stages of a completely different model of acutely protein-deficient weanling guinea pigs vaccinated with *Mycobacterium tuberculosis*[5]. Blood concentrations of hormones and cytokines represent spillover from the extravascular compartment and may be regarded as reflective, although probably not representative, of concentrations at extravascular sites of action[3,6]. High levels of glucocorticoids and IL-10, therefore, are positioned temporally to initiate depressed inflammatory capacity in the early stages of acute malnutrition, and a potent triad including the glucocorticoids, IL-10 and TGF-β is likewise positioned to serve in a sustaining role. It is unknown whether TGF-β might also contribute to the initiation of malnutrition-associated depression in inflammatory immune competence.

TGF-β exerts potently anti-inflammatory and immune suppressive effects impacting cells of both the innate and adaptive arms of immune defence[7], and this cytokine is regarded as a dominant peripheral mediator of anti-inflammatory self-tolerance[8,9]. The objective of this investigation was to determine if the blood TGF-β concentration rises during the early stages of acute pre-pubescent malnutrition in metabolically distinct murine models known to depress inflammatory immune competence[10–12]. Additionally, a confirmatory experiment was conducted assessing blood levels of TGF-β in the advanced stages of acute weanling malnutrition.

Materials and methods

Animals and facilities

Male and female C57BL/6J mice were obtained from an in-house breeding colony. Caging and housing conditions were exactly as described previously[2,12], and this investigation

Abbreviation: TGF, transforming growth factor.

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was approved by the Animal Care Committee of the University of Guelph in accordance with the guidelines of the Canadian Council on Animal Care.

Diets and feeding protocols

At 18 d of age, the animals were weaned, individually housed and given free access for 1 d to a complete purified diet that is described elsewhere[13]. At 19 d of age, the mice were randomly allocated to one of three experimental groups. An age-matched control group was given *ad libitum* access to the aforementioned complete purified diet. An energy-restricted group consumed the complete diet in restricted daily quantities determined by calculations that relate *ad libitum* food intake (g food/g body weight) to chronological age in the weanling mouse[12]. Finally, a low-protein group was given free access to a purified diet formulated to contain 0·6 % crude protein by replacement of most of the nitrogen source of the complete diet by maize starch[10]. These malnutrition protocols reproduce the critical features of the paediatric human pathologies of marasmus via the restricted intake protocol and incipient kwashiorkor via the low-protein protocol[2,3,12].

Experimental design

Two experiments were performed. In the first experiment, ten animals were included in each dietary group and blood samples were taken at day 3, representative of the early stages of weight loss[2]. In the second experiment, intended to be confirmatory of a previous report pertaining to the advanced stages of malnutrition[3], sample sizes consisted of ten animals in the age-matched control group and eight animals in both malnourished groups, and blood was taken after 14 d. Equal numbers of males and females were included in all dietary groups from both experiments.

Blood sampling procedure

Orbital plexus blood samples were collected under CO_2 anaesthesia as described previously[3]. Platelet-poor plasma was collected and stored at −80°C as described previously[3].

Plasma transforming growth factor-β bioassay

The assay was based on the reduction of the proliferative activity of Mv1Lu mink lung cells (ATCC CCL-64) exposed to TGF-β and was performed as described in detail elsewhere[3]. Recombinant human TGF-β1 (BD Biosciences, San Jose, CA, USA; catalogue #559119) was used to generate standard curves (average R^2 0·98), and only linear portions of the curves were used. The intra-assay CV averaged 4·6 %, and the detection limit was 46 pg/ml. Each plasma sample was analysed in triplicate.

Carcass composition

Carcasses were stored at −80°C to await analysis of DM and total lipid content as described previously[10].

Statistical analysis

Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC, USA) for Windows (version 9.0), and a pre-determined upper limit of probability of $P \leq 0{\cdot}05$ was applied for statistical significance. Data were subjected to a one-way ANOVA followed, if justified by Tukey's Studentised Range test. Datasets not exhibiting a normal distribution were transformed. Where transformation attempts were unsuccessful, data were subjected to the Kruskal–Wallis test (χ^2 approximation) followed, if justified, by Wilcoxon two-sample testing.

Results

Distinct weight loss pathologies were elicited by the malnutrition protocols

Growth indices for the two experiments are shown in Table 1. Within each experiment, initial body weights did not differ among groups. Moreover, the age-matched control groups exhibited food intakes and carcass composition outcomes comparable to those reported previously for C57BL/6J

Table 1. Initial and final body weights, food intakes and carcass compositions

(Mean values with their standard errors)

	Dietary group			
Index	C	LP	R	SEM
Day 3				
Initial body weight (g/mouse)	7·9	8·2	8·2	0·09
Final body weight (g/mouse)	9·6[a]	7·4[b]	6·9[b]	0·09
Food intake (g/mouse per d)	2·2[a]	1·6[b]	1·1[c]	0·03
Food intake (g/g body weight per d)*	0·17[a]	0·13[b]	0·10[c]	0·001
Carcass DM (g/100 g wet weight)†	29·5[a]	29·0[a]	27·8[b]	–
Carcass lipid (g/100 g wet weight)	8·9[a]	6·6[b]	3·9[c]	0·14
Day 14				
Initial body weight (g/mouse)‡	8·4	8·5	8·4	0·006
Final body weight (g/mouse)§	18·3[a]	6·2[b]	6·4[b]	0·01
Food intake (g/mouse per d)‖	2·6[a]	1·2[b]	0·9[c]	–
Food intake (g/g body weight per d)§	0·15[a]	0·10[b]	0·07[c]	0·03
Carcass DM (g/100 g wet weight)¶	32·0[a]	29·1[b]	27·8[b]	–
Carcass lipid (g/100 g wet weight)	9·6[a]	4·8[b]	2·2[c]	0·17

C, group that consumed *ad libitum* the complete diet; LP, group that consumed *ad libitum* an isoenergetic low-protein diet; R, group that consumed the complete diet in restricted daily quantities.

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$) according to Tukey's Studentised Range test, unless a different statistical procedure is indicated.

* From ANOVA of squared-transformed data. Mean values are square roots of squared means.

† Kruskal–Wallis test of Wilcoxon rank sums that were as follows: C, 187; LP, 188; R, 90.

‡ From ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

§ From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

‖ Kruskal–Wallis test of Wilcoxon rank sums that were as follows: C, 215; LP, 98·5; R, 37·5.

¶ Kruskal–Wallis test of Wilcoxon rank sums that were as follows: C, 202; LP, 83·5; R, 65·5.

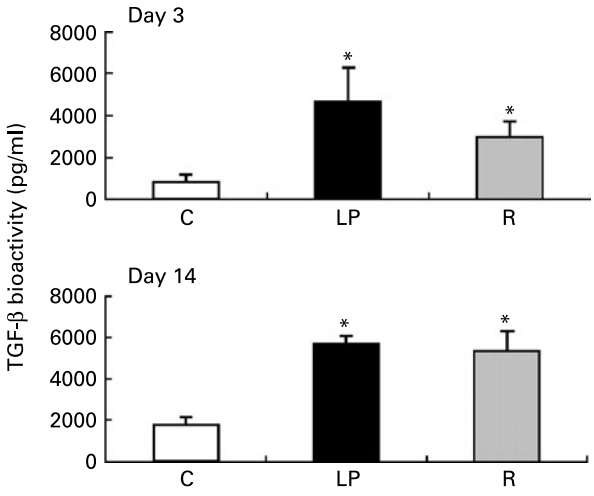

Fig. 1. Plasma transforming growth factor (TGF)-β bioactivity. Weanling C57BL/6J mice, initially 19 d old, were fed *ad libitum* a complete purified diet (group C, age-matched controls), or the complete diet in restricted daily quantities (group R) or were given free access to an isoenergetic low-protein diet (group LP). Equal numbers of males and females contributed to samples sizes of ten in all dietary groups (upper panel, day 3), or to sample sizes of ten (group C) and eight in each malnourished group (lower panel, day 14). Bars represent mean values and the SEM is shown in each case. Within each experiment, data were analysed by one-way ANOVA. Day 3: means are antilogs of natural log-transformed means; diet $P=0{\cdot}0004$, pooled SEM = 0·16. Day 14: diet $P=0{\cdot}0001$, pooled SEM = 339. Bars marked with an asterisk (*) differ from their corresponding age-matched control group ($P\leq 0{\cdot}05$) according to Tukey's Studentised Range test.

weanlings consuming the complete purified diet[2,3,12]. At the end of each experiment, the body weights and food intakes of the malnourished groups were lower than their corresponding age-matched control group. Also, each malnourished group exhibited a lower carcass fat level than its corresponding age-matched control, and the restricted intake protocol induced a greater decrement in carcass fat, and hence in carcass energy, than the low-protein protocol. Overall, the loss of both fat and lean tissue produced by the two malnutrition protocols was comparable to the outcomes reported previously when inflammatory immune competence was shown to be depressed in the same experimental systems[10–12].

Plasma transforming growth factor-β bioactivity

Plasma TGF-β bioactivities are shown in Fig. 1. Samples from all groups exhibited plasma bioactivities that exceeded the detection limit of the assay. Plasma TGF-β bioactivities found in the age-matched control groups were consistent with concentrations reported previously in plasma samples from healthy adolescent and young adult mice[3,14]. In relation to the main objective of this investigation, plasma TGF-β bioactivities were elevated in both malnourished groups after only 3 d of weight loss, and the high blood levels of this cytokine were sustained into the advanced stages of protein and energy deficit.

Discussion

This investigation reveals that the plasma concentration of TGF-β rises early in response to acute pre-pubescent deficits of protein and energy, and demonstrates that the high levels of this anti-inflammatory cytokine are maintained into the advanced stages of weight loss. The findings pertaining to the later stages of malnutrition confirm previous reports[2,5]. This outcome was apparent in two metabolically distinct murine models that mimic the critical features of paediatric marasmus and incipient kwashiorkor[2,3,12]. Thus, a potently anti-inflammatory triad of soluble mediators, namely the glucocorticoids, IL-10 and TGF-β, emerges when this work is considered together with previous reports[2–5] and provides a basis for understanding both initiation and maintenance of depressed inflammatory competence in acute pre-pubescent malnutrition. More broadly, this investigation provides new experimental underpinning for the concept[1] that malnutrition-associated immune depression is part of a regulated pathophysiology, the antithesis of a biologically trivial disintegration of immunological capacities.

Attempts to achieve a biologically meaningful assessment of TGF-β concentrations in blood and other tissue fluids are complicated by the existence of three mammalian isoforms of this protein mediator, designated β1–3,[15] and by the fact that much of the cytokine circulates in latent form[16]. Only the β1 isoform is detectable in the blood of the healthy mouse or of mice subjected to the low-protein protocol used herein, whereas both β1 and β2 isomers are reported at high levels in the blood of mice subjected to our food intake restriction protocol[3]. It is important, therefore, that the three mammalian isoforms exhibit indistinguishable immunological influences, at least *in vitro* [3], and equal biological activity in the bioassay used herein[15]. Moreover, TGF-β immunoassays[17] and bioassays[5,17] frequently include an initial activation step that releases the cytokine from its latent form, thereby inflating the measurement that is interpreted as biologically active TGF-β. However, this assay strategy was not used in the present investigation. To the extent that blood cytokine concentrations reflect extravascular levels[3,6], therefore, the TGF-β concentrations determined herein are directly relevant to the levels of biologically active cytokine that target cells would encounter *in vivo*.

Numerous possibilities merit investigation as contributors to high circulating levels of TGF-β in acute malnutrition. In this connection, findings pertaining to the small intestinal epithelium and lamina propria of acutely malnourished infants and children lend no support to the possibility of an increase in the rate of cytokine synthesis[18]. However, a much more extensive cellular survey is needed as virtually all cells can produce TGF-β[16]. Likewise, reduced turnover must be considered in parallel with findings reported for other circulating proteins of acutely malnourished children, e.g. IgG class Ig[19] and some acute-phase proteins[20,21]. An intriguing possibility pertains to the extracellular matrix, which appears to serve as a reservoir for TGF-β[16,22]. Proteolytic degradation of the extracellular matrix liberates TGF-β from its latent complex, thereby coupling matrix turnover with the activation of TGF-β[22]. Thus, by exaggerating a normal physiological phenomenon, the catabolic metabolism of acute malnutrition may support a rapid and sustained elevation in blood and tissue fluid concentrations of a potent anti-inflammatory cytokine in its active form. In this connection, protein- and energy-deprived rats exhibit decreased soluble and insoluble collagen skin content in comparison to healthy controls[23].

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The glucocorticoids, IL-10 and TGF-β comprise a network of partially redundant anti-inflammatory hormonal mediators effective against diverse innate and adaptive immune defence elements and widely regarded as the backbone of peripheral tolerance[7–9,24,25]. The present investigation completes the picture with respect to this potent network in acute prepubescent malnutrition. It is now apparent, from this investigation and other reports[2–4], that the three component mediators rise early and are sustained in unison throughout the progression of weight loss. In turn, this points to the recent proposition of a coordinated and purposeful immune regulation in which the inflammatory form of competence is supplanted by an anti-inflammatory form that is tolerant of the self-antigens catabolically released in quantity during wasting malnutrition[1,3]. This tolerance-centred model emphasises the adaptive benefit of a reduced risk of autoimmune disease while acknowledging the cost in terms of susceptibility to infectious disease. Other anti-inflammatory mediators, e.g. IL-35[24], can be accommodated within the tolerance model, and their participation can be anticipated in the non-inflammatory immune competence of acute prepubescent malnutrition.

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